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FREEZING AND DESICCATION OF MOUSE TUMOURS*

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[WITH SPECIAL PLATE]

Gye and his colleagues (Gye, 1949 ; Gye, Begg, Mann, and Craigie, 1949 ; Mann, 1949b, 1949c ; Mann and Dunn, 1949) published a series of experiments on the propagation of mouse tumours, first by frozen and then by frozen and desiccated tumour tissues. As the result of these experiments they arrived at the following conclusions: (*a*) that freezing alone is sufficient to kill tumour cells, and more effective still is freezing followed by drying ; and (*b*) that the newly induced tumours must have been initiated by a virus, since frozen and frozen-and-dried tumour material is able to induce tumours on inoculation.

In view of the interesting experimental evidence and the far-reaching conclusions based on it, experiments were undertaken (*a*) to repeat the observations described by Gye *et al.* with their own and other tumours, and (*b*) to test their claim to have obtained evidence of a cancer virus.

The technique followed throughout the experiments was, in essence, that described by the above authors. The pump employed was the "Speedivac" Model 2 two-stage rotary vacuum pump, which gives a vacuum of 0.00001 mm. Hg and has a displacement of 48 litres a minute (W. Edwards & Co., Ltd.). No estimate of the weight of the dried material was made by Gye *et al.* so far as can be judged from their publications. They report no attempts to dry their material to a constant weight, and we know of none. Gye (1948–9, page 13) states that Craigie's apparatus "dries a gramme of finely divided tumour tissue in less than an hour," and that "the tissues have been dried to dust." In another experiment Gye (1948–9, page 14) refers to "fine dry scales of tissue" after drying for 130 minutes. As these were the only criteria of dryness described by him we accepted and adopted them. In most of the present experiments 5.3% glucose solution was used as a diluent fluid in quantities either equal to, or up to three times, the weight of the tumour material, usually 1 g. The drying-time varied according to the amount of tissue and diluent in the container. The desiccation process was usually carried on for 3½ hours. The dried material was reconstituted in 5.3% glucose solution or in 1 in 500 neutral solution of cysteine or in a fluid composed of equal quantities of 5.3% glucose solution and cysteine solution, all added in amounts from three to five times the original volume of the tumour-cell suspension. The resulting suspension was injected into the mice by various routes in quantities varying from 0.1 to 0.5 ml.

It was found that there was no difficulty in repeating the results of Gye *et al.*, both those of freezing and those of freeze-drying the various sarcoma tissues employed in the present experiments.

1. Experiments with Mouse Sarcomata

The results are set out in Table I. The failure to obtain tumours in the first two experiments with the 37S tumour may have been due partly to faulty technique or to the condition of the tumour tissue, while the negative result with the Crocker sarcoma may have been due to a temporary breakdown in the supply of solid CO_2, when the temperature in the insulated box rose to $-12°$ C. The negative results with these two tumours will be referred to in the section on histology.

2. Experiments with Mouse Breast Carcinomata

Employing the same technique we found that the breast carcinomata do not stand up to the conditions of the experiment in the same way as do the sarcomata. The results both of freezing and of freezing followed by desiccation of mouse mammary carcinomata are presented in Table II.

It will be seen that while the results with the carcinomata are not as good as those with the sarcomata, they suffice to confirm the observations of Mann (1949b) and Mann and Dunn (1949). Altogether seven experiments were carried out—three with spontaneous C3H breast carcinomata and four with transplantable C3H breast carcinomata. While it must be admitted that the number of experiments is too small from which to draw any final conclusion about a possible difference between transplantable and spontaneous

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TABLE I.—*Freezing, and Freezing Followed by Desiccation, of Different Sarcomata*

Strain	Tumour	Diluent	Time Frozen	Desiccation Time	Method of Inoculation	Mice		No. of Tumours	Time of Appearance	Time of Observation	Histology	Remarks
						Sex	No.					
(C57 x A) F_2	37 sarcoma	None	30 days	Not done	Subcutaneous	M, F	10	0	—	11 weeks	Not done	—
,,	,,	,,	30 ,,	,,	Intrapleural	M, F	4	0	—	7 ,,	,,	—
,,	,,	,,	30 ,,	,,	Intraperitoneal	M, F	5	5	1–3 weeks	7 ,,	,,	—
,,	,,	,,	74 ,,	3½ hours	Subcutaneous	M	10	5	2–3 ,,	5 ,,	,,	5·3% glucose added before desiccation
C57 ..	C48 sarcoma	5·3% glucose	51 ,,	Not done	,,	M	3	3	5–8 days	3 ,,	Done	—
,,	,,	,,	51 ,,	,,	Intraperitoneal	M	3	3	5–8 ,,	3 ,,	,,	—
,,	,,	,,	51 ,,	3½ hours	Subcutaneous	M	3	2	2–5 weeks	7 ,,	,,	Desiccated tissue reconstituted in cysteine
,,	,,	,,	51 ,,	3½ ,,	Intraperitoneal	M	2	2	3 ,,	7 ,,	,,	,, ,,
,,	,,	,,	27 ,,	3½ ,,	Subcutaneous	M	18	18	1–2 ,,	3 ,,	,,	Desiccated tissue reconstituted in 5·3% glucose
C3H ..	C3H sarcoma	,,	7 ,,	3½ ,,	,,	M	7	4	2–3 ,,	4 ,,	,,	,, ,,
,,	,,	,,	10 ,,	2 ,,	,,	M	12	11	1 week	2 ,,	,,	Desiccated tissue reconstituted in equal volumes of 5·3% glucose and cysteine
,,	,,	,,	12 hours	1 hour	,,	M	6	6	4–5 days	2 ,,	,,	Desiccated tissue reconstituted in 5·3% glucose
RIII ..	RIII (No. 4) sarcoma	,,	87 days	3½ hours	,,	M, F	12	12	2 weeks	4 ,,	,,	,, ,,
(C57 x A) F_2	Crocker sarcoma	,,	107 ,,	Not done	,,	M, F	12	0	—	5 ,,	,,	—

TABLE II.—*Freezing, and Freezing Followed by Desiccation, of Mammary Carcinomata.*

Strain	Tumour	Diluent	Time Frozen	Desiccation Time	Method of Inoculation	Mice		No. of Tumours	Time of Appearance	Time of Observation	Histology	Remarks
						Sex	No.					
C3H ..	C3H spontaneous breast carcinoma	None	13 days	Not done	Subcutaneous	M	12	2	2–3 weeks	12 weeks	Not done	—
,, ..	,,	Physiological saline	13 ,,	,,	,,	M	6	4	4–8 ,,	14 ,,	,,	—
,, ..	C3H transplantable breast carcinoma	None	30 ,,	,,	,,	M	12	0	—	12 ,,	,,	—
,, ..	,,	,,	30 ,,	,,	Intrapleural or intraperitoneal	M	6	0	—	12 ,,	,,	—
,, ..	,,	5·3% glucose	19 ,,	,,	Intraperitoneal	M	11	0	—	17 ,,	Done	—
,, ..	,,	None	74 ,,	3½ hours	Subcutaneous	M	12	0	—	12 ,,	Not done	Desiccated tissue reconstituted in 5·3% glucose
,, ..	C3H spontaneous breast carcinoma	5·3% glucose	11 ,,	3½ ,,	,,	M	3	1	8 weeks	12 ,,	Done	,, ,,

carcinomata in the resistance to freezing and drying, attention might be drawn to the results of Klinke (1940) and Cramer (1930), who similarly observed that spontaneous mammary carcinomata showed a greater resistance to cold (−196° C.) than transplantable breast carcinomata.

It is not altogether surprising that the sarcomata should withstand the treatments of freezing and of freeze-drying better than the carcinomata. The carcinomata as a whole are derived from more highly differentiated tissues than the sarcomata, and it would not be unreasonable to expect them to be more susceptible to adverse conditions. Certainly in our experiments there were more visible changes, suggesting cell damage, to be found microscopically in the case of the carcinomata than in the sarcomata ; it may not be unreasonable to associate this fact with the experimental results.

Centrifugation of Frozen and Frozen-desiccated Material

Having satisfied ourselves that the results described by Gye *et al.* can be repeated at will, not only with the same but also with different tumours, we undertook an experiment to test whether an active agent or virus could be separated from the tumour cells or whether the tumour-inducing activity is associated with the tumour cells themselves.

Suspensions of minced tumour tissues which had been previously frozen, or frozen and dried, and then reconstituted were centrifuged at 3,000 to 7,000 times gravity for periods of time varying from two to five minutes. Such centrifugation is insufficient to remove viruses from fluid suspensions, and it is reasonable to expect that any virus present would remain in the supernatant fluid. Our aim was to obtain at least a partial separation of virus from the tumour cells, and to test the two resulting fractions for activity by inoculation. The results of inoculation of the sediment and of the supernatant fluids are shown in Tables III and IV.

As can be seen from these tables, tumours were induced in each case with the sediment obtained after centrifugation of the various tumour suspensions, and in no case were tumours observed following inoculation of the water-clear supernatant.

Table IV demonstrates the greater susceptibility to the combined treatment of cold and drying that was noticed in the mammary carcinomata as compared with the sarcomata. Only one tumour was induced by the carcinomata, and in this case by the sediment.

Thus from these centrifugation experiments it is quite clear that the power of tumour suspensions, whether frozen or frozen and then dried, to induce tumours remains with

TABLE III.—*Centrifugation of Frozen and Frozen-desiccated Sarcomata.*

Strain	Tumour	Diluent	Time Frozen	Desiccation Time	Centrifugation	Method of Inoculation	Mice: Sex	Mice: No.	No. of Tumours	Time of Appearance	Time of Observation	Histology	Remarks
(C57 x A) F_2	37 sarcoma	None	30 days	Not done	3,000 × g. 5 minutes	Subcutaneous supernatant	M, F	11	0	—	11 weeks	Not done	Centrifuged in 5·3% glucose
,,	,,	,,	30 ,,	,,	,,	Subcutaneous sediment	M, F	10	4	3–4 weeks	11 ,,	,,	,,
,,	,,	5·3% glucose	18 ,,	3½ hours	7,000 × g. 3 minutes	Intraperitoneal supernatant	M	7	0	—	9 ,,	Done	Desiccated tissue resuspended in cysteine
,,	,,	,,	18 ,,	3½ ,,	,,	Intraperitoneal sediment	M	9	3	5–13 days	9 ,,	,,	,,
C57 ..	C48 sarcoma	,,	51 ,,	Not done	3,500 × g. 5 minutes	Subcutaneous supernatant	M	2	0	—	12 ,,	,,	—
,,	,,	,,	51 ,,	,,	,,	Intraperitoneal supernatant	M	4	0	—	12 ,,	,,	—
,,	,,	,,	51 ,,	,,	,,	Subcutaneous sediment	M	2	2	5–9 days	3 ,,	,,	—
,,	,,	,,	51 ,,	,,	,,	Intraperitoneal sediment	M	4	4	9–13 ,,	3 ,,	,,	—
,,	,,	,,	51 ,,	3½ hours	7,000 × g. 5 minutes	Subcutaneous supernatant	M	6	0	—	12 ,,	,,	Desiccated tissue reconstituted in cysteine
,,	,,	,,	51 ,,	3½ ,,	,,	Subcutaneous sediment	M	9	6	4–5 weeks	12 ,,	,,	,,
C3H ..	C3H sarcoma	,,	30 ,,	3½ ,,	7,000 × g. 2 minutes	Subcutaneous supernatant	M	6	0	—	12 ,,	,,	Desiccated tissue reconstituted in 5·3% glucose
,,	,,	,,	30 ,,	3½ ,,	,,	Subcutaneous sediment	M	12	10	2 weeks	4 ,,	,,	,,
(C57 x A) F_1	Crocker sarcoma	,,	22 ,,	3½ ,,	7,000 × g. 3 minutes	Subcutaneous supernatant	M, F	16	0	—	16 ,,	,,	Desiccated tissue reconstituted in equal quantities of glucose and cysteine
,,	,,	,,	22 ,,	3½ ,,	,,	Subcutaneous sediment	M, F	22	16	1–3 weeks	9 ,,	,,	,,

TABLE IV.—*Centrifugation of Frozen and Frozen-desiccated Breast Carcinomata.*

Strain	Tumour	Diluent	Time Frozen	Desiccation Time	Centrifugation	Method of Inoculation	Mice: Sex	Mice: No.	No. of Tumours	Time of Appearance	Time of Observation	Histology	Remarks
C3H ..	C3H transplantable breast carcinoma	None	30 days	Not done	7,000 × g. 5 minutes	Subcutaneous supernatant	M	12	0	—	11 weeks	Not done	Centrifuged in 5·3% glucose
,,	,,	,,	30 ,,	,,	,,	Subcutaneous sediment	M	12	0	—	11 ,,	,,	,,
,,	,,	5·3% glucose	38 ,,	3½ hours	7,000 × g. 3 minutes	Subcutaneous supernatant	M	9	0	—	18 ,,	Done	Desiccated tissue resuspended in equal volumes of glucose and cysteine
,,	,,	,,	38 ,,	3½ ,,	,,	Subcutaneous sediment	M	9	0	—	18 ,,	,,	,,
,,	,,	,,	25 ,,	3½ ,,	7,000 × g. 2 minutes	Subcutaneous supernatant	M	7	0	—	21 ,,	Not done	,,
,,	,,	,,	25 ,,	3½ ,,	,,	Subcutaneous sediment	M	10	1	14 weeks	21 ,,	,,	,,

the tumour cells. Everything points to the activity being associated with the cellular material, alive or dead, as it is never found in the clear cell-free supernatant, as might reasonably be expected if the action were the results of a virus. A reasonable deduction from this observation is that some tumour cells may survive the treatment and thus be responsible for the transmission of tumours. Histological study greatly strengthens this deduction.

Histological Investigations

Gye *et al.* (1949) stated that the results of their histological examinations of random samples of cell suspensions had failed to support the idea of cell survival. Dried tissues examined by them "showed only a mass of debris and shrunken nuclei." In their papers there is little information about histological appearance, and in none of them do they present any illustration, nor is there any description, of the appearances of frozen and dried tumour material fixed *after* reconstitution. Mann and Dunn (1949) reproduce a photograph of a mammary carcinoma fixed immediately after freezing followed by desiccation but *before* reconstitution, although there is no figure to illustrate the same tissue fixed *after* reconstitution.

In each of the present experiments the minced tumour tissues were fixed in Bouin's fluid, stained with haematoxylin and eosin, and examined microscopically: (1) before freezing, (2) after freezing in 5.3% glucose, (3) after freezing followed by drying *before* reconstitution, and (4) after combined freezing and drying *after* reconstitution in glucose or cysteine.

The results are shown in Figs. 1–20 (see Plate). It is difficult to detect any differences in the appearance of many of the sarcoma cells before and after freezing (see Figs. 4 and 5). Similarly, after freezing and desiccation followed by reconstitution, the suspensions of sarcoma cells had an appearance similar to that of the same suspensions before freezing and drying (see Figs. 1 and 2, 4 and 7, 9 and 10, 11 and 13, and 14, 18, and 20).

However, in no case could intact cells be found after freezing and drying but *before* reconstitution: this observation agrees entirely with that described by Gye *et al.* (1949) (see Figs. 6, 12, and 19) in revealing "only a

mass of debris and shrunken nuclei." The addition of any of the mentioned diluents to the frozen and desiccated material *before* fixation in Bouin's fluid creates an amazing and dramatic effect on the fixed tissues (see Figs. 6 and 7, 12 and 13, and 19 and 20). Many of the tumour cells are, to all appearances, intact, resembling in their entirety the tumour cells before the treatment.

How it can have come about that Gye *et al.* (1949) and Mann and Dunn (1949) omitted this step is not clear; but it is a matter for conjecture whether the failure to observe the appearances of frozen and dried tissue *after* reconstitution might not have been one of the reasons for their interpretation of the results.

While the microscope cannot with certainty reveal if tumour cells are alive or dead, the appearance of tumour-cell suspensions obtained before and after freezing, or freezing followed by drying, strongly suggests that some of the tumour cells might have been alive and have been responsible for the induction of tumours either with frozen or with frozen-and-dried material. From the appearance of the tumour cells there is no evidence that they are dead. The biological results in every case support the inferences which might reasonably be drawn from the microscopical appearances. Thus in every case in which the tumours were obtained by inoculation with the treated material the microscope suggested that at least many cells might be alive: certainly they seemed very little altered when compared with the fresh tumour-cell suspension; while, in those cases in which the biological tests were negative, histological examination showed considerable cell injury or even loss of cell structure. This was particularly well shown in the experiments with the Crocker sarcoma (Fig. 3) and with 37S (Fig. 8). In the Crocker suspension, frozen for 107 days, no intact cells were seen and no tumours were induced, while the 37S tumour suspension, which presented profound cellular changes, gave fewer tumours than suspensions of other tumours treated in a similar manner. Again, in the case of C3H mammary carcinomata similar changes, indicative of cell injury, could be seen both in the frozen and in the frozen-desiccated material, which goes hand in hand with the erratic results obtained with the C3H mammary carcinomata, especially the transplantable one (see Figs. 15, 16, and 17).

To sum up, it might in general be said that, viewing the results of the microscopical studies as a whole, the material which presents little histological evidence of cell damage can be relied upon to induce tumours more readily than that which has a damaged appearance.

Discussion

(a) Freezing and Drying

If there has been no difficulty in repeating the experiments of Gye *et al.* (Table V), the same cannot be said of accepting their interpretation—namely, that these experiments indicate a virus to be the cause of the successful transmission of tumours by frozen-and-dried material. Before discussing the reasons for the inability to agree with Gye's conclusions, experiments in this field by previous workers will be described and discussed. It is felt that a careful perusal of some of the available experiences of previous workers should in itself command the greatest caution in accepting the conclusions reached by Gye *et al.*, because in the literature there is ample evidence that extremes of cold, far beyond anything employed by Gye, can be successfully resisted by some organisms without jeopardizing life.

Gye *et al.* were obviously very much impressed with the inferences which they had drawn from experiments

TABLE V.—*Summary of Results*

Tumour	Freezing Alone	Freezing and Desiccation	Centrifugation	No. of Mice	No. of Tumours
37 sarcoma ..	+	–	–	19	5
,, ..	–	+	–	10	5
,, ..	+	–	Supernatant	11	0
			sediment	10	4
,, ..	–	+	,,	7	0
				9	3
C48 sarcoma ..	+	–	–	6	6
,, ..	–	+	–	23	22
,, ..	+	–	Supernatant	6	0
			sediment	6	6
,, ..	–	+	,,	6	0
				9	6
Crocker sarcoma	+	–	–	12	0
,, ..	–	+	Supernatant	16	0
			sediment	22	16
C3H sarcoma..	+	–	–	25	21
,, ..	–	+	Supernatant	6	0
			sediment	12	10
RIII (No. 4) sarcoma ..	+	–	–	12	12
C3H spontaneous breast carcinoma ..	+	–	–	18	6
,, ..	–	+	–	3	1
C3H transplantable breast carcinoma ..	+	–	–	29	0
,, ..	–	+	–	12	0
,, ..	+	–	Supernatant	12	0
			sediment	12	0
,, ..	–	+	,,	16	0
				19	1

+ Denotes procedure carried out. – Denotes procedure not carried out.

of Ida Mann (1949a), in which she found that embryonic tissues of mice do not survive exposure to freezing at –79° C. for one hour. But surely there was nothing new in this, as many previous observers had already published work of a similar nature. Thus Gaylord (1908) was not able to obtain subcutaneous transplantation of mouse embryo epithelium after exposure to –180° C. Lambert (1912) observed that chick embryos developed after exposure to –5° C. for 10 days, and that chick embryo heart was killed after one minute at –74° C., but survived at –20° C. for five minutes. Simonin (1931) found that fragments of mouse and rat embryo survived for five days at –5° C., but were killed at –15° C.

There are numerous publications on the storage of tumour tissues at low temperatures for periods varying up to two years, and a successful transplantation of such tissues into animals. The methods employed by different authors were extremely variable: long exposure to only a few degrees of cold, short exposure to very low temperatures, and single or repeated exposures to various temperatures have all been described for various types of tumours, usually with a great measure of success.

Michaelis (1905) was the first to observe the survival of Jensen rat sarcoma after exposure to liquid air for 30 minutes. Ehrlich (1907) reported survival of carcinoma cells after storage at –8° C. for two years and after 48 hours at 38° C. Salvin-Moore and Walker (1908) exposed a transplantable mouse mammary carcinoma to liquid air for 30 minutes, and succeeded in transplanting it. Salvin-Moore and Barret (1908) observed similar results after exposure of mouse sarcoma and carcinoma to liquid air for 20 minutes. Gaylord (1908) confirmed these findings. Cramer (1930) froze finely minced tissues of seven different mammalian tumours in CO_2 and found that sarcomata grew and carcinomata failed to grow on transplantation following this treatment. Some of the tumours (37S) induced tumours after freezing and thawing repeated eight times. Auler (1932) found that Ehrlich mouse carcinoma grew on transplantation after an exposure to –73° C. for 25 minutes, and Jensen rat sarcoma after five minutes at –73° C., and that Flexner–Jobling carcinoma failed to grow.

Barnes and Furth (1937) and Breedis. Barnes, and Furth (1937) successfully transplanted leukaemia of mice after exposure to −70° C. for 56 days and also a similarly treated sarcoma induced by benzpyrene. Breedis and Furth (1938) reported the survival of various tumours (sarcomata and carcinomata) after storage at −79° C. for 400 days. Klinke (1937) demonstrated successful transplantation of many mice and rat tumours after direct exposure to −196° C. for periods up to 47 hours. The only observed difference between tumours treated in this way and the controls was the delay in tumour appearance after freezing. Mider and Morton (1939) reported that Walker rat carcinoma and 37S and 180 mouse sarcomata grew on subcutaneous transplantation after exposure to −79° C.

Klinke (1940) succeeded in demonstrating growth in tissue culture of Ehrlich mouse sarcoma after exposure to −253° C. lasting 10 minutes, and of Jensen rat sarcoma after two days at −196° C. Although intact cells could be seen in tissue culture of a transplantable breast carcinoma after exposure to these temperatures, growth was not obtainable. Klinke concluded that exposure to low temperatures gives no basis for the existence of a virus in mammalian tumours because of the results of vital staining and growth in tissue cultures. Subsequent to Gye's papers, Craigie (1949a, 1949b, 1949c) with proper caution suggested that there may be some cell survival following freezing of tumour cells in a mixture of dextrose and 40% glycerol. Of all these results, Klinke's (1940) obtained after exposure of tumour cells to low temperature are perhaps the most pertinent, as he clearly demonstrated the survival and growth *in vitro* of tumour cells which had been so exposed. This property of survival following exposure to cold is not limited to tumour cells, as can be seen from the quoted literature.

Not only does the literature abound in examples of the survival of tumour cells after extremes of freezing, but there are many references to the ability of normal cells, tissues, and organisms to survive such treatment. Luyet and Gehenio (1938a) reviewed the experiments on survival of unicellular and other primitive organisms after freezing. Heilbrunn (1943) also reviewed experiments on the resistance of single-celled and multicellular animals and plants to very low temperatures. It is now known that some organisms can withstand temperatures close to the absolute zero, while others are killed at temperatures above the freezing-point of water, but the rate at which an organism is cooled and the rate at which it is brought back to higher temperatures is known to affect the ability of the organism to withstand the cold to which it is subjected.

De Jong (quoted by Heilbrunn) observed that trypanosomes are able to resist exposure to −190° C. Rahm (1922) demonstrated that nematodes, rotifers, and tardigrades placed in water cooled gradually could be brought to the temperature of liquid hydrogen (−253° C.) without death, but that they died when brought to that temperature suddenly. Luyet and Gehenio (1938b) reported survival of plant cells after exposure to liquid air, and Luyet and Hartung (1941) demonstrated survival of common vinegar eels (*Anguilla aceti*) after immersion in liquid air (−190° C.), preceded by immersion in 30% ethylene glycol solution, and rapid rewarming in water at 30° C. They reported that in one experiment they had observed a vinegar eel after freezing in liquid air give birth to a live offspring, which survived two weeks. Breedis and Furth (1938) reported survival of chicken epithelium after exposure at −79° C., Mider and Morton (1939) the survival of squamous epithelial cells of homologous skin grafts in the rat, and Briggs and Jund (1944) survival of normal mouse skin after exposure to −79° C. for one hour. Webster (1944) described survival of human skin on grafting after exposure to −72° C. for periods up to 17 days, and, what is even more interesting, after drying in the frozen state. Blumenthal and Walsh (1950) have recently demonstrated the survival of guinea-pig thyroid and parathyroid in auto-transplants exposed to −190° C.

There is besides a large body of work on the survival of spermatozoa following extremes of low temperatures, and even after freezing and drying. Davenport (1897) possibly was the first to show the resistance of human spermatozoa to low temperatures. Jahnel (1938) demonstrated the resistance of human spermatozoa to −196° C. and even to −269.5° C. The spermatozoa showed motility after 52 hours at −196° C., also after exposure to −79° C. for 40 days or to −269.5° C. for three hours. Weber (1938) observed motility of bull spermatozoa after 96 hours at 0° C. Luyet and Hodapp (1938) found that frog spermatozoa, and Shaffner, Henderson, and Card (1941) that fowl spermatozoa, are resistant to exposure to liquid air. Shettles (1940) observed that human spermatozoa show motility after exposure to −79° C. for up to two months, and to −196° C. (liquid nitrogen) or −269° C. (liquid helium) for five minutes. He also noted individual variation in the sensitivity of various samples of human spermatozoa to these three temperatures. Morphologically they appeared to be in good condition, and continued their motility for several hours; after freezing and drying the morphology of spermatozoa was preserved in his hands, but there was no motility. Hoagland and Pincus (1942) confirmed this observation and reported survival of 67% human spermatozoa after exposure to −195° C. Polge, Smith, and Parkes (1949) observed survival of fowl spermatozoa diluted in equal parts of Ringer's solution containing 40% glycerol at −79° C. for 20 minutes, and after freeze-drying for three hours at −25° C. in 20% glycerol in Ringer's solution.

Altogether from previous work it is crystal clear, therefore, that normal and tumour tissues can withstand freezing, even if embryo tissues cannot: yet in the face of the published evidence Gye (1949) definitely states: "The final conclusion we have reached from these studies is that malignant cells, like embryonic cells, are readily killed by extreme cold. . . ."

(b) Virus Claim

Gye (1949), Gye *et al.* (1949), Mann (1949b, 1949c), and Mann and Dunn (1949) claimed that their successful induction of tumours, after preliminary freezing or freeze-drying of tumour-cell suspension, was due to a tumour-inducing virus. Having decided that freezing and freeze-drying killed the cancer cell, they deduced that the ability of the frozen tumours to induce fresh tumours must have been due to a virus, the activity of which had not been destroyed. So far as can be ascertained from their published papers, this was a deduction from a limited observation. For Gye *et al.* carried out no experiments to separate the supposed virus from the cellular material, nor did they test biologically for the presence of life in the tumour cells after freezing or freeze-drying. But it is an established fact that successful implantation of cellular material is a sound test for cell life, and the simplest interpretation of their results is that the material which they were transplanting contained living cells. There was no need to invoke the presence of a virus.

Subsequent to the original papers (Gye, 1949; Gye *et al.*, 1949) Craigie (1949c) differed from the original view about desiccation as expressed therein. He considers the possibility that a few cells (about 1 in 1,000,000) survive desiccation. But it is still not clear, at least to us, that he

considers, as we do, that the surviving cells are responsible for the tumours induced with frozen-dried material.

Apparently Gye *et al.* made no attempt to look for a virus with the electron microscope, which in these days is perhaps the method *par excellence* for the detection of viruses. For what the observation is worth, we have found that such tumours as have been examined in low-cancer strains, whether induced or spontaneous, are free of any virus-like particles under the electron microscope, whereas in high-breast-cancer strains the electron microscope has consistently revealed the presence of particulate matter mainly of some 250 Å (Passey, Dmochowski, Astbury, and Reed, 1947; Passey, Dmochowski, Astbury, Reed, and Johnson, 1948, 1950; Passey, Dmochowski, Reed, and Astbury, 1950), which is always associated with the Bittner agent.

To sum up: first, in none of our centrifuge experiments were tumours induced with clear supernatants free from cells, while with few exceptions the sediments, in which apparently intact cells were always to be found, regularly induced tumours, which is clear evidence that the tumour-inducing activity was always associated with the tumour cell, and was not separable from it; secondly, the microscopical evidence provides further support for the belief that some of the cells had survived, in that, after the various treatments of freezing and freeze-drying, many intact cells were to be found which in appearance were very little different from the untreated minced material. It seems to us, in view of the available evidence, that there is no need to invoke a virus as the active agent.

It must, however, be admitted that a final decision on whether the cells are viable and are responsible for the results can be reached only after an attempt to grow tumour cells *in vitro* after freeze-drying. Experiments along these lines have been carried out in this department in collaboration with Dr. I. Lasnitzki, from the Strangeways Laboratory, Cambridge. The results of these experiments are reported in the paper which immediately follows this communication.

Summary and Conclusions

Minced tumours, both sarcomata and carcinomata, retain their power to induce fresh tumours after freezing and subsequent desiccation under the experimental conditions described by Gye *et al.*

Centrifugation at slow speeds of tumour tissues similarly treated, together with our histological studies, indicates that living cells, and not a virus, are responsible for the results.

We should like to express our thanks and appreciation to Mr. W. Blackledge, in charge of the photography department of Leeds University, for his help and great care in taking photographs of the histological specimens. We also wish to acknowledge the help given by Mr. Kenneth Morton in the preparation and staining of the histological specimens.

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CULTIVATION IN VITRO OF FROZEN AND DESICCATED MOUSE TUMOUR TISSUES*

BY

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[WITH SPECIAL PLATE]

In order to arrive at a final decision on whether the conclusions of the experiments described in the preceding paper (Passey and Dmochowski, 1950), that freezing and dessication do not necessarily kill tumour cells, were sound, it was decided to put the matter to the test of tissue culture. It was, however, fully realized that it is not as delicate a test as the biological one of animal inoculation, as the conditions of growth *in vitro* are, in general, not as good as those *in vivo*.

Method

The tumour used in the present experiments was the C3H sarcoma described in the original experiments by Gye *et al.* (1949). The tumour tissues were minced,

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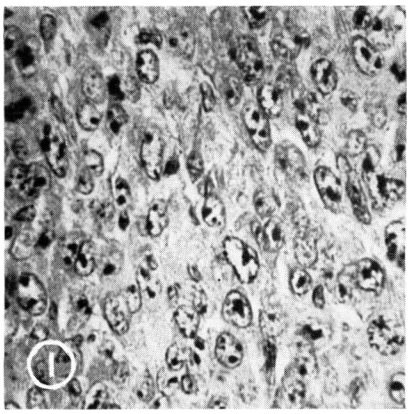


FIG. 1.—Crocker sarcoma. Minced tumour tissue before freezing. H. and E. (× 500.)

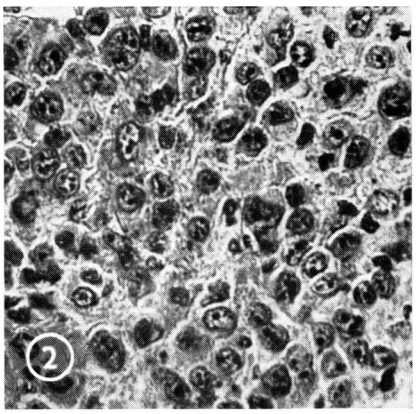


FIG. 2.—Same Crocker sarcoma. Minced tumour tissue suspension in 5.3% glucose; frozen in CO_2 at −79° C. for 22 days; desiccated; reconstituted in equal quantities of 1:500 neutral solution of cysteine and 5.3% glucose. H. and E. (× 500.)

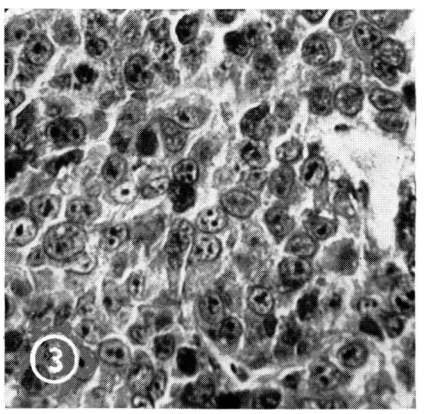
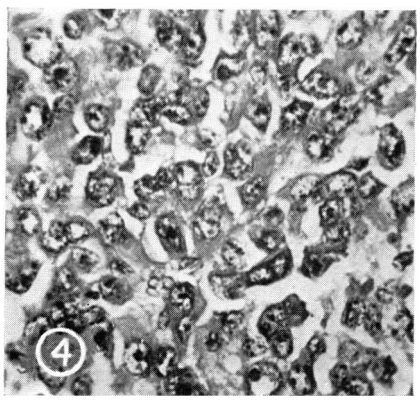
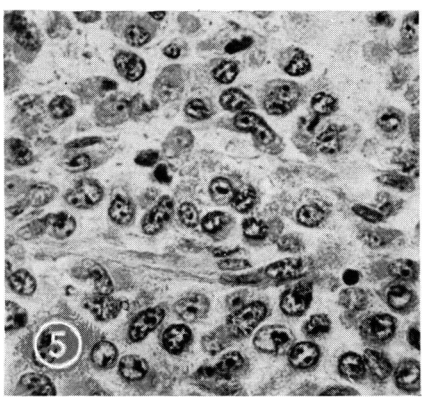
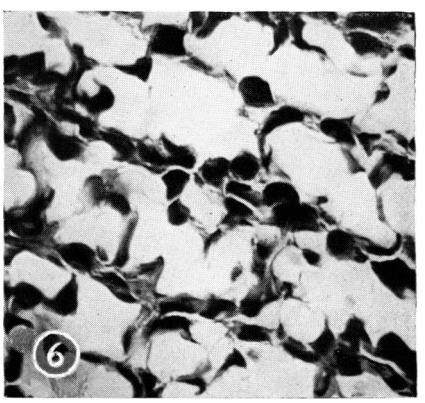
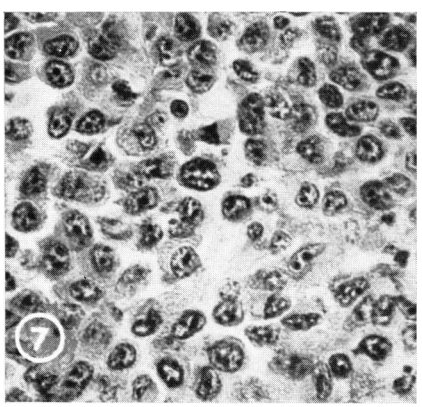


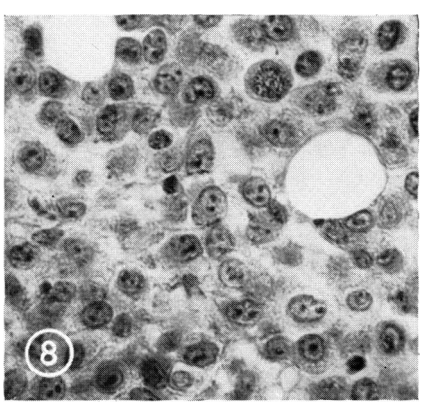
FIG. 3.—Same Crocker sarcoma. Minced tumour tissue, suspension in 5.3% glucose; frozen in CO_2 at −79° C. for 22 days; desiccated; reconstituted in cysteine and glucose. Some damaged cells seen. H. and E. (× 500.)











FIGS. 4–8.—37S sarcoma. Minced tumour tissue. H. and E. (× 500.)

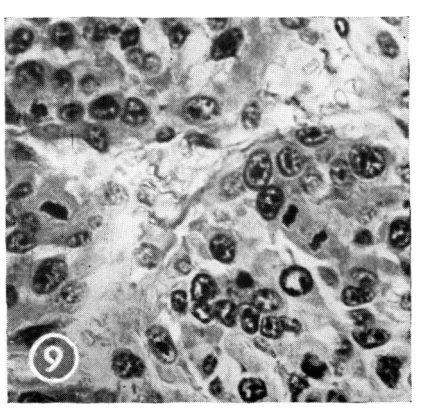
FIG. 4.—Before freezing.

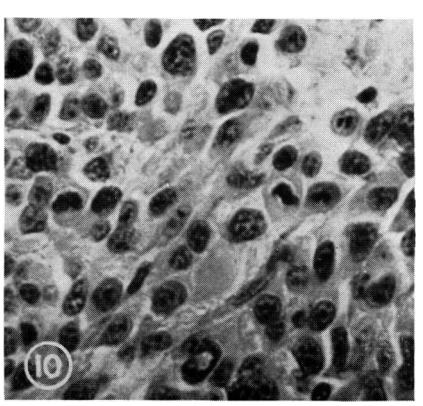
FIG. 5.—Suspension in 5.3% glucose; frozen in CO_2 at −79° C. for 18 days.

FIG. 6.—Suspension in 5.3% glucose; frozen in CO_2 at −79° C. for 18 days; desiccated.

FIG. 7.—Suspension in 5.3% glucose; frozen at −79° C. for 18 days; desiccated; reconstituted in 5.3% glucose.

FIG. 8.—Suspension in 5.3% glucose; frozen in CO_2 at −79° C. for 18 days; desiccated; reconstituted in 5.3% glucose. Some damaged cells seen.





FIGS. 9–10.—C48 (I.C.R.F.) methylcholanthrene-induced sarcoma. H. and E. (× 500.)

FIG. 9.—Tumour tissue before freezing.

FIG. 10.—Minced tumour tissue; suspension in 5.3% glucose; frozen in CO_2 at −79° C. for 51 days; desiccated; reconstituted in cysteine.

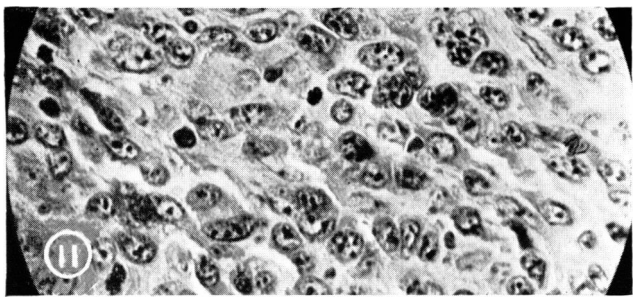


FIG. 11.—C3H (I.C.R.F.) sarcoma. Tumour tissue before freezing. H. and E. (× 500.)

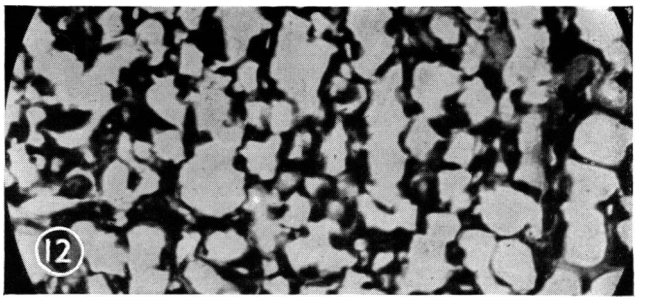


FIG. 12.—Same C3H (I.C.R.F.) sarcoma. Minced tumour tissue; suspended in 5.3% glucose; frozen at −79° C. for 29 days; desiccated. H. and E. (× 500.)

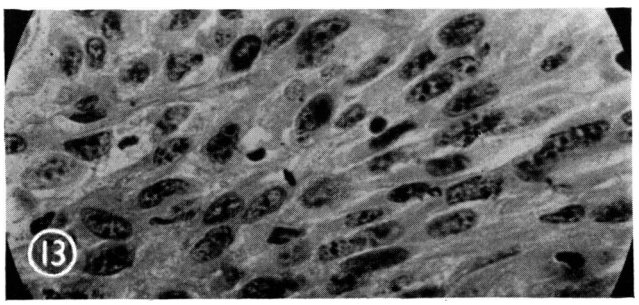


FIG. 13.—Same C3H (I.C.R.F.) sarcoma. Minced tumour tissue; suspended in 5.3% glucose; frozen at −79° C. for 29 days; desiccated; reconstituted in 5.3% glucose. H. and E. (× 500.)

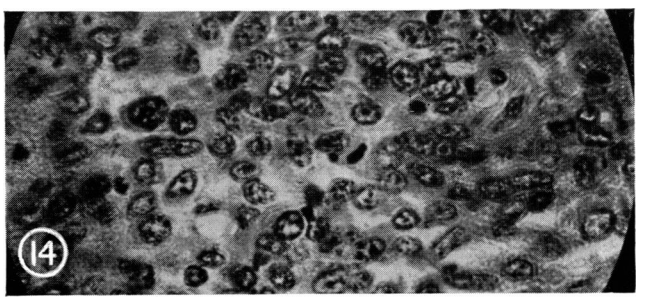


FIG. 14.—Same C3H (I.C.R.F.) sarcoma. Minced tumour tissue; suspended in 5.3% glucose; frozen at −79° C. for 29 days; desiccated; reconstituted in 5.3% glucose; centrifuged for two minutes at 7,000 times gravity. Sediment. H. and E. (× 500.)

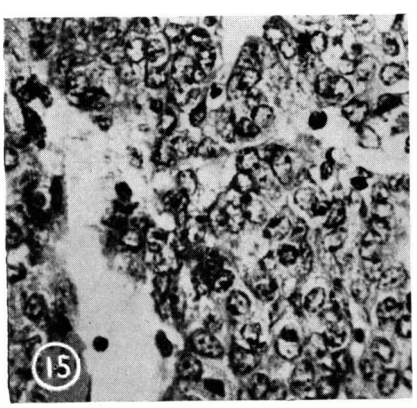


FIG. 15.—C3H transplantable breast carcinoma tissue before freezing. H. and E. (× 500.)

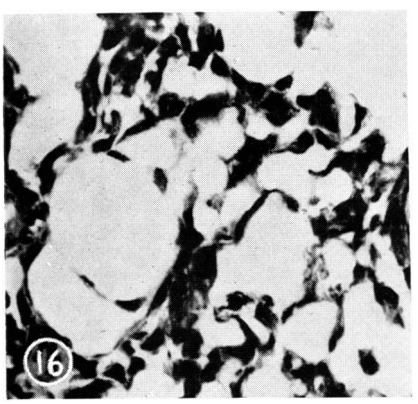


FIG. 16.—Same C3H transplantable breast carcinoma. Minced tumour tissue; suspension in 5.3% glucose; frozen in CO_2 at −79° C. for 38 days; desiccated. H. and E. (× 500.)

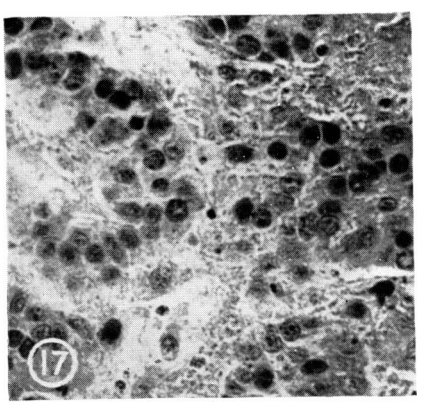


FIG. 17.—Same C3H transplantable breast carcinoma. Minced tumour tissue; suspension in 5.3% glucose; frozen in CO_2 at −79° C. for 38 days; desiccated; reconstituted in cysteine and glucose; centrifuged for three minutes at 7,000 times gravity. Sediment. Damaged cells seen. H. and E. (× 500.)

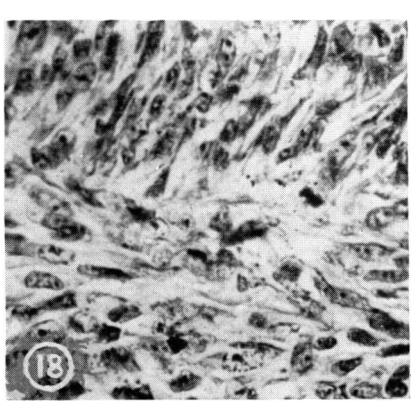


FIG. 18.—RIII (No. 4) sarcoma; not frozen and not desiccated. Control. H. and E. (× 500.)

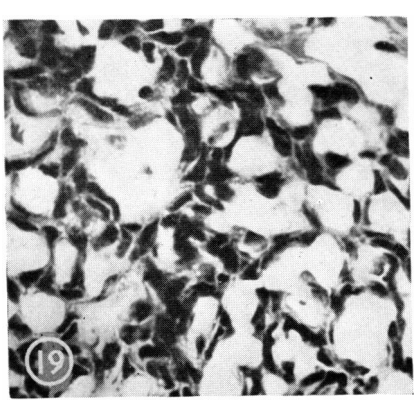


FIG. 19.—Same RIII (No. 4) sarcoma. Frozen in 5.3% glucose at −79° C. for 87 days; desiccated for 3½ hours. H. and E. (× 500.)

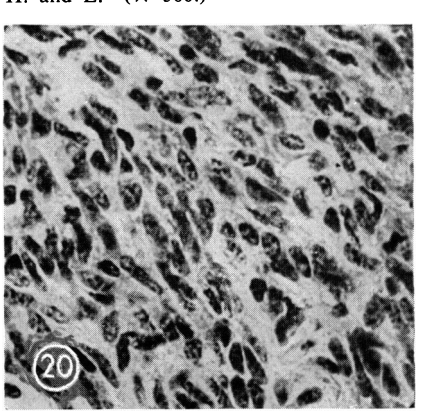


FIG. 20.—Same RIII (No. 4) sarcoma. Frozen in 5.3% glucose at −79° C. for 87 days; desiccated for 3½ hours; reconstituted in 5.3% glucose. H. and E. (× 500.)